

Appendix E

COMPARISON BETWEEN WILD TYPE AND MUTATED RECEPTORS: TDAG & GPR35

A. TDAG8

293 cells were plated-out on 150mm plates at a density of 1.3×10^7 cells per plate, and were transfected using 12 μ g of the respective DNA and 60 μ l of Lipofectamine Reagent (BRL) per plate. The transfected cells were grown in media containing serum for an assay performed 24 hours post-transfection.

A commercially-available, 96-well Adenylyl Cyclase Activation Flashplate™ was used (NEN: #SMP004A). First, 50 μ l of the standards for the assay were added to the plate, in duplicate, ranging from concentrations of 50pmol to zero pmol cAMP per well. The standard cAMP (NEN: #SMP004A) was reconstituted in water, and serial dilutions were made using 1xPBS (Irvine Scientific: #9240). Next, 50 μ l of the stimulation buffer (NEN: #SMP004A) was added to all wells. Next, the 293 cells transfected with the respective cDNA (CMV¹ or TDAG8) were harvested 24 hours later (assay detection in serum media). The media was aspirated and the cells washed once with 1xPBS. Then, 5ml of 1xPBS was added to the cells along with 3ml of cell dissociation buffer (Sigma: #C-1544). The detached cells were transferred to a centrifuge tube and centrifuged at room temperature for five minutes. The supernatant was removed and the cell pellet was resuspended in an appropriate amount of 1xPBS to obtain a final concentration of 2×10^6 cells per milliliter. The detection buffer containing the tracer cAMP was prepared. In 11ml of detection buffer (NEN: #SMP004A), 50 μ l (equal to 1 μ Ci) of [¹²⁵I]cAMP (NEN: #SMP004A) was added. Following incubation, 50 μ l of this detection buffer containing

¹ This is the vector used for expression, such that the control was the vector, CMV, excluding the receptor TDAG8 (or GPR35 in the example below).